Carbohydrate Polymers 87 (2012) 177–185

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Pectinase treatments on technical fibres of flax: Effects on water sorption and mechanical properties

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ARTICLE INFO

Article history:
Received 6 April 2011
Received in revised form 18 June 2011
Accepted 20 July 2011
Available online 2 August 2011

Keywords:
Flax
Cellulosic fibre
Nanoindentation
Pectate-lyase
Retting
Tensile properties
Water vapour sorption

ABSTRACT

This study is focused on enzymatically upgrading the functional properties of flax fibres. Green flax fibres were treated with a polygalacturonase and a pectate lyase (PaL) and their properties were compared with dew-retted fibres. Morphological observations, vapour-sorption analyses and mechanical measurements showed that PaL-treatment was able not only to mime retting in terms of bundle division, but also to improve the mechanical properties of technical fibres. Conversely, these properties were shifted down after the polygalacturonase treatment, mainly due to the presence of contaminating glycanases. At the level of the elementary fibres, nanoindentation data indicated the highest stiffness of the secondary wall for PaL-treated fibres. The tensile properties exhibited equal, but moderate values of the Young's modulus (~37 ± 14 GPa) and breaking strength (~650 ± 300 MPa) for retted and PaL-treated fibres; we hypothesize an impact of the growth conditions on the fibre chemical structure with an excess of matrix pectins compared to the amount of glucomannan coating the cellulose microfibrils.

1. Introduction

In flax stem, non-lignified cellulosic fibres provide structural support by absorbing mechanical stress. By using these fibres in reinforced composites, their properties, i.e. tensile strength and stiffness and low density, are transferred to the composite (Bledzky & Gassan, 1999; Mohanty, Misra, & Hinrichsen, 2000). However, due to fibre defaults induced at different steps of the transformation processing, the mechanical properties of technical fibres are far from that of fibres in their native bundle (e.g. Romhany, Karger-Koesis, & Czigany, 2003). Another fragility factor arises from dew-retting which largely relies on soil fungi to colonize the stems and to degrade the cell-wall of the cortex cells by releasing hydrolases such as pectinases, xylanases, arabinases, but also glucanase and cellobiohydrolases (Brown & Sharma, 1984). The consequences are that, over the several weeks of retting, the fibre cell-walls might have been remodelled and weakened due to the many enzymes secreted by the fungi.

One alternative strategy to improve the retting process includes the use of pectinases that catalyze pectin depolymerisation within the cortex tissues and the tri-fibre junctions (Morvan, Jauneau, Voreux, Morvan, & Demarty, 1990). Pectinases applied on flax stems or technical fibres consisted generally of commercial mixtures, among which were Flaxzyme (Van Sumere & Sharma, 1991), Viscozyme (Zhang, Henriksson, & Johansson, 2000) or Pectinex AR (Stuart et al., 2005). An advantage of these enzymatic processes was to produce fibres with high yield and tactile qualities. However, the fibre strength was generally decreased due the presence of contaminating hydrolases. Alternatively, using pectin lyase or pectate-lyase solutions at high pH would reduce the hydrolase activities whose optimal pH is generally within the range of 3–6 (Jauneau, Morvan, Morvan, Demarty, & Devauchelle, 1986; Jauneau, Morvan, Fenyo, & Demarty, 1988). Thus, using an alkaline pectate lyase arising from transgenic B Lichiniformis (provided without cellulase by BioPrep 3000), Akin et al. (2007) showed that the strength of thread/technical fibres was higher than when using an acidic polygalacturonase enriched solution.

It has been shown (Sharma, 1988) that the main limiting factor of retting consists of calcium pectates. These pectates are located in the cell junctions of all tissues including the fibre bundles, as well as in the external tangential wall of the epidermis (Andème-Onzighi, Girault, His, Morvan, & Driouich, 2000; Jauneau et al., 1992). Concerning the secondary wall of fibres, it is essentially made up of oriented, highly crystalline microfibrils embedded in encrusting non-cellulosic polysaccharides (NCPs). The NCPs contain the pectic matrix-polymers, homogalacturonan (HGA) and rhamnogalacturonan (RG-I), which could be extracted with hot HCl (Alix, Goimard, Morvan, & Baley, 2009). HGA is an α-1,4 homopolymer of

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doi:10.1016/j.carbpol.2011.07.035

D-galacturonic acid while the RG-I consists of a backbone alternating galacturonic acid and L-rhamnose (α-1,2 linked) on which long side chains (mainly β-1,4 galactans) are branched (Gorshkova & Morvan, 2006). On the other hand, there are polysaccharides tightly bound to cellulose, which can be solubilized in boiling alkali. The latter extract contains glucomannans (GM; containing alternating blocks of β-1,4 glucan and β-1,4 mannan) as the principal hemicelluloses (McDougall, 1993; Van Hazendonk, Reinerink, De Waard, & Van Dam, 1996), but also pectic HGA and RG-I enriched in galactose (Girault et al., 1997; Gurjanov, Ibragimova, Gnezdilov, & Gorshkova, 2008; Mooney, Stolle-Smits, Schols, & de Jong, 2001).

The aim of the present paper was to investigate the effects of pectinases on the chemistry of the fibres decorticated from green stems of flax and to compare their physico-chemical and mechanical properties to those of the corresponding dew-retted samples. In a first part, we compared polygalacturonase and pectate-lyase treated fibres by evaluating (i) the amount of released sugars and (ii) the mechanical properties of the technical fibres. In a second part, we further characterized pectate-lyase treated and dew-retted fibres in terms of chemical composition, sorption behaviour and mechanical properties.

2. Experimental

2.1. Materials

Flax fibres, Hermes variety grown in Normandy in 2004 (variety designated as H4), were provided by Dehondt Technologies Company (Notre-Dame de Gravenchon, France). The trial was sown on April 14th (with 50 units of nitrogen). After pulling (August 26th, sum of temperature: 2000 °C; sum of rain fall: 550 mm), plants were laid on the field for drying for 1 week, giving, after scutching, green technical fibres, designated as H4V. Other plants were submitted to dew-retting for 6 weeks, giving, after scutching, technical retted fibres, named H4R. It is worth noting that, contrary to the elementary fibres, the term "technical fibre" refers to a group of fibres either in a complete bundle such as in H4V or in a partly divided bundle such as in H4R.

2.2. Enzymatic treatments

Two commercial enzymes (i) Lyvelin (containing 11,000 units (U)/g polygalacturonase (PG), diluted 1v:100v in 0.1 M sodium succinate pH 5.0) and (ii) Peclyve (PaL, containing 375 U/g pectate lyase (PaL), diluted thrice (v/v) in 50 mM sodium carbonate + 1 mM CaCl2 pH 8.2) were provided by Lyven SA (Cagny, France). Enzymatic treatments were performed using 1 g H4V samples according to Lyven procedures, providing technical fibres designated as H4V-PG and H4V-PaL for polygalactase and pectate lyase handlings, respectively. One unit was defined as 1 μmol of reducing sugar released in 1 min at 30 °C.

The controls were run in buffer without enzyme. Each experiment was replicated three times. After treatment, the samples were abundantly rinsed in de-ionised water and air-dried for further tests, providing technical fibres designated as H4V-PG and H4V-PaL for polygalactase and pectate lyase handlings, respectively.

2.3. Optical microscopy

Cross-sections were hand-cut from the middle part of 2–3 average stems, coloured (carmin 40:10 g/L and methyl green: 1 g/L) and observed with a Leica optical microscope. The equivalent diameter of fibres was estimated as Deq = (lf + Lf)/2, lf and Lf being their small and large dimensions (in μm). When soaked in Calcofluor White M2R (Sigma–Aldrich, 0.01% w/v), a fluorescent probe which interacts with cellulose, the microscope was coupled with a Zeiss filter set (excitation filter, 350–410 nm wavelength; barrier filter, 470 nm wavelength).

2.4. Immunolabelling procedures

Two monoclonal primary antibodies, JIM5 and JIM7, that are specific to partially methyl-esterified homogalacturonans (HGA), were used according to the protocol of Knox, Linstead, King, Cooper, and Roberts (1990). Briefly, fibres were laid in a blocking solution of milk and incubated in droplets of JIM5 or JIM7 antibodies (diluted 1v:5v). After washing, the samples were treated for 2 h at 25 °C with the secondary antibody conjugated to fluorescein isocyanate (FITC) (anti-rat IgG diluted 1v:50v) and observed using an Axioskop microscope (Zeiss) equipped with an epifluorescence filter set (excitation filter, 485 nm wavelength; barrier filter, 515–565 nm wavelength). Observations were made at various times depending on the intensity of the labelling to avoid the saturation response of fluorescence as a function of time. Non-specific binding was controlled by the omission of the primary antibody or using primary and secondary antibodies onto boiling-alkali treated fibres in order to remove all surface non cellulosic polymers. The data were expressed after subtracting the values of control fibres (without primary antibody or alkali treated) obtained for the same amount of observation time.

2.5. Extraction of polysaccharides and sugar analyses

The H4R and H4V-PaL technical fibres were pre-treated at 100 °C (3× 2 h) with water and then in EDTA-Na_2 as previously described (Goubet et al., 1995). The cleaned fibres were then successively treated with 0.02 M HCl and 1.5 M NaOH/100 mM $NaBH_4$ (once 1 h 100 °C + twice in H_2O for 1 h at 100 °C) in order to release the polysaccharides (named EH and EOH, respectively) that encrusted and coated the cellulose microfibrils (Charlet et al., 2007). The residues were treated by sulfuric acid (H_2SO_4 for 1 h then diluted to 1 M and heated 2 h at 110 °C) to dissolve the microfibrils of cellulose and quantify their amount. Three independent series were run for each sample. Total sugars and galacturonic acids were colourimetrically assayed (Blumenkrantz and Asboe-Hansen, 1973; Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and sugar composition determined after methanolysis and silylation and separation by gas chromatography (Goubet et al., 1995).

2.6. Water sorption measurements

Water vapour sorption kinetics were studied at 25.0 ± 0.1 °C on 5–20 mg samples by using an automated electronic microbalance (Cahn D200 with a mass resolution of 0.1 μg) in an automated gravimetric dynamic vapour sorption system DVS1 Advantage (Surface Measurement Systems Ltd.). Two types of sorption analysis were performed, kinetics and isotherms.

As for kinetics aspects, two stages were considered as a function of time (i) a fast one (from 10 min to 60 min, as long as the mass variation, relative to the equilibrium mass gain, remained ≤0.2) and (ii) a slower last stage which corresponded to the higher gain of mass; thus, two diffusion coefficients D_1 and D_2 were determined (Gouanvé et al., 2006).

In the case of flax fibres, isotherms usually have a sigmoid shape and can be divided into three different regions as a function of water activity a_w, so as it was more convenient to consider the Park model (Park, 1986). It corresponded to a multi sorption mode that could be divided into three steps: (1) Langmuir sorption, (2) Henry's law and (3) water clustering. The water content, C, at the equilibrium state was used to build the sorp-

tion isotherm as a function of water activity a_w according to Eq. (1).

$$C = \frac{A_L \cdot b_L}{1 + b_L \cdot a_w} \cdot a_w + k_H \cdot a_w + n \cdot K_a \cdot k_H^n \cdot a_w^n \quad (1)$$

Langmuir's terms, A_L (Langmuir capacity constant) and b_L (Langmuir affinity constant) had an influence at low water activity ($a_w < 0.1$). Henry's solubility coefficient, k_H, defined the slope of the isotherm when $0.1 < a_w < 0.8$. The values K_a (equilibrium constant for the clustering reaction) and n (mean number of water molecules per cluster) could be linked to the equilibrium state ($a_w > 0.8$) corresponding to water-aggregate formation.

2.7. Mechanical properties

Tensile tests were performed on technical fibres (gauge length was 75 mm) using an Instron 5543 testing machine (5 N load cell) as previously described (Alix, Philippe, et al., 2009). Tensile tests on elementary fibres (gauge length 10 mm) were performed using a universal tensile testing machine (NFT 25-704, ASTM D 3379-75; 2 N load cell) as reported by Baley (2002). All tests (minimum 20, up to 100 samples) were carried out until rupture with a cross-head speed of 1 mm min^{-1} at constant temperature (23 °C) and hygrometry (48% relative humidity). Young's modulus (E), failure strength (σf) and failure strain (εf) were calculated taking into account the average section of technical or elementary fibres as previously described (Baley, 2002).

2.8. Nanoindentation

Fibre samples were embedded in LR-White resin as previously described (Andème-Onzighi et al., 2000). Blocks of 6 mm thickness were used after their surface topography was minimized by using an ultramicrotomic apparatus. Then, blocks were mounted on aluminum cylinders using Superglue©(at the block bottom). Indentation tests (Nanoindenter XP, MTS Nano Instruments) were run at room temperature (23 ± 1 °C) with a continuous stiffness measurement (CSM) technique. An oscillating force at controlled frequency and amplitude was superimposed onto a nominal applied force so that the material would respond with a displacement phase and amplitude (Li & Bhushan, 2002). The elastic modulus ($E - n$) was estimated from the experimental curves, as has been previously described (Bourmaud & Baley, 2009). A three-side pyramid (Berkovitch) diamond indenter was used, whose area function calculating the contact area from the contact depth was calibrated by using a standard sample. After the indenter made contact with the surface, it was driven into the material with a constant strain rate, 0.05 s^{-1} to a depth of 120 nm; the load was held at maximum value for 60 s; then, the indenter was withdrawn with the same rate as loading until 10% of the maximum load was reached. A 3 nm amplitude and 70 Hz oscillation were chosen for the CSM parameters. Matrices of 20 × 20 indents were carried out with 1 μm between each indent.

3. Results and discussion

3.1. Morphology of fibres

In the 2004 harvest, stems of Hermes variety (H4) were relatively short with a technical length of 65 ± 5 cm and small diameter (1.2 ± 0.3 mm as measured in the medium part of 50 stems). Such values suggested a limited growth when compared to Hermes cultivated in 2002 (fibre technical length being 80 cm; Charlet et al., 2007). According to Sharma and Faughey (1999), stem length and diameter are dependent on flax cultivar, but more importantly on the availability of nutrient in the soil, crop management and weather conditions.

The organization and shape of fibres was studied from the middle section of H4, because the fibres located in the middle part of the stem generally yield the best fibres in terms of morphology and tensile properties (Charlet et al., 2007). They were organized in 38 ± 2 bundles (total average area 5500 ± 500 μm^2) containing 26 ± 9 fibres into 3–4 layers of 5–6 aligned fibres (Fig. 1A). The equivalent diameter (Deq) of elementary fibres was estimated around 16 ± 5 μm in the medium part of stems whose diameter was representative of the mean. The fibre section was rather hexagonal with a ratio lf/Lf of 0.82 ± 0.14, but their filling appeared uncompleted. The porosity (defined as the ratio of the lumen surface area to the fibre surface area was estimated by Charlet, 2008) to be larger in 2004 (6.8 ± 3.5%) than in 2002 (3.4 ± 1.9%).

After scutching, the equivalent diameters (Deq) of H4V and H4R technical fibres were estimated to be 135 ± 35 μm and 85 ± 20 μm, respectively.

Table 1
Mean tensile properties of technical fibres. The gauge length was 75 mm and the cross-head displacement rate 1 mm min^{-1}. The diameter value (⌀) corresponded to the mean of those of the tested samples (number = n). σf, εf, E, corresponded to the stress, strain at the sample failure (f) and Young's modulus respectively. H4V, H4R, corresponded to green and retted technical fibres. H4V-PG, H4V-PaL corresponded to H4V treated with polygalacturonase and pectate lyase, respectively.

	H4V	H4R	H4V-PG	H4V-PaL
σf(MPa)	305 ± 120[1]	310 ± 120[1]	325 ± 115[1]	470 ± 165
εf(%)	1.3 ± 0.4[b]	1.1 ± 0.4[1,a]	1.9 ± 0.9[2]	1.4 ± 0.5
E (GPa)	31 ± 12[2,b]	32 ± 12[2,a]	22 ± 12[1]	37 ± 15
⌀ (μm)	135 ± 33	85 ± 20	95 ± 32	76 ± 16

[1,a]Significantly different from the value of H4V-PaL or H4V-PG, respectively (Student's test $P < 0.01$).
[2,b]significantly different from the value of H4V-PaL or H4V-PG, respectively (Student's test $P < 0.05$).

3.2. Enzymatic treatments

3.2.1. Efficiency of the enzymes

After treatment of H4V with pectate lyase (PaL, pH 8.0) and polygalacturonase (PG, pH 5.5), about 70–90 mg of total sugars (TS) were released over the 100–200 mg of contaminating cortex matter generally measured in 1 g of non-retted fibres (e.g. Morvan et al., 1990; Van den Oever, Bas, Van Soest, Melis, & Van Dam, 2003). The amount of released uronic acids (UA) was lower with PaL (25–35 mg) than with PG (30–45 mg), indicating that not all the same pectins were removed with PG or PaL.

3.2.2. Mechanical properties of the technical fibres after retting or enzymatic treatments

Despite taking precautions to minimize variation arising from the sampling and test procedures, tensile mechanical property data remained highly variable (Table 1). No matter the number of samples tested from 19 to 90, the variation coefficient was comprised between 35 and 39% for the failure stress (σf) and was even larger for the elastic modulus. Even with the large scattering of the experimental values, PaL treated samples displayed the highest σf values, close to those reported by Bos, Van den Oever, and Peters (2002) for technical fibres when the clamping length was larger than 25 mm. On the other hand, σf values of H4V, H4R and H4V-PG were significantly lower than this target value. Their tensile modulus was also lower than that of H4V-PaL; only the difference between H4V-PG and H4V-PaL was highly significant. Hence, these mechanical data indicate that, in H4V-PaL, enough pectins remained in the fibre cell-junctions to transfer the tensile stress to the elementary fibres

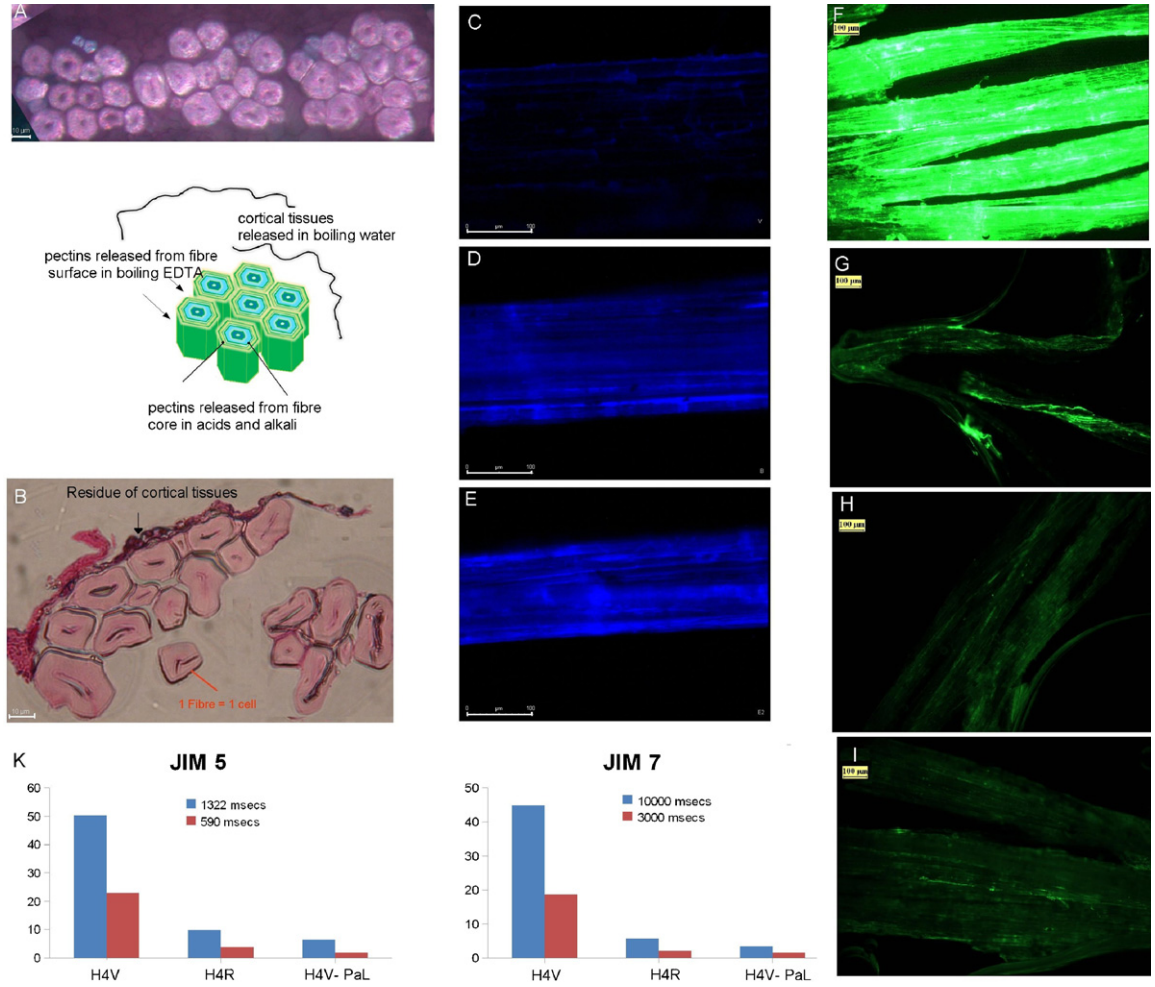

Fig. 1. Morphological and immunocytochemical observations of technical fibres. (A) Fibre bundle in H4V section and its schematic representation. (B) H4R samples. Note the heterogeneity in the number of fibres still associated together. (C–E) Calcofluor epi-fluorescence at the surface of H4V (C), H4R (D) and H4V-PaL (E) samples. (F–H) JIM5 immunofluorescence at the surface of H4V (F), H4R (G) and H4V-PaL (H) samples. (I) Immunofluorescence control when omitting JIM5 antibody. (K) Semi-quantitative estimation of JIM5 and JIM7 labellings.

within the divided bundle. From these first mechanical data, PaL treatment appeared to be an interesting alternative to dew retting.

The origin of the low tensile value was probably different in H4V and H4R or H4V-PG. The H4V sample exhibited low properties, due to the lowest amount of the load-bearing cellulose and its largest diameter (Baley, 2002). The lower values of failure stress and elastic modulus of H4V-PG compared to H4V-PaL might originate from the contamination of commercial PG by glucanase activity that might partially degrade the surface cellulose microfibrils and decrease their global rigidity. H4R was mainly affected through σf values, which means that the pectins in fibre junctions have been weakened during dew-retting. Compared to H4R, H4V-PG exhibited higher elastic deformation. The differences might originate from the nature and swelling of the inter-fibre cements which depend on the amount of calcium pectate, i.e. the limiting factor of the retting (Sharma, 1988). After PG incubation in sodium succinate buffer, a maximum of sodium pectate was removed as indicated by the high value of UA released in the incubation buffer. The remaining pectins, possibly neutralized by sodium, would better swell and consequently would facilitate the inter-fibre cement deformation and the slipping of the fibres along the 75 mm length.

In the following, we restricted our study to H4V-PaL (having the best tensile properties), H4V and H4R samples.

3.3. Impact of the PaL treatment on the surface of the technical fibres

3.3.1. Fluorescent microscopy

Both H4R and H4V-PaL samples fixed calcofluor, a probe with high affinity for cellulose, while a faint signal characterized H4V (Fig. 1C–E). In H4V, fibre cellulose was masked by the pectic cements adhering to the bundle, as well as by the cortex debris. Conversely, the H4V surface was enriched in HGA, as seen by the labelling with the two antibodies, JIM5 (as illustrated in Fig. 1F–H) and JIM7 (Fig. 1K). After retting or PaL treatment, the JIM5 and JIM7 labellings significantly decreased indicating that a significant part of the HGA surface had been released from the technical fibres.

3.3.2. Composition of surface pectins

Retted fibres consisted of more and less divided bundles with surface spotting of contaminating cortex debris (Fig. 1B). This debris was enriched in HGA and RG-I with short side-chains of galactose and arabinose (named surface pectins) and could be eliminated with boiling water (e.g. Morvan et al., 1990). Besides, pectins from fibre junctions could be extracted with boiling EDTA (Goubet et al., 1995). As a whole, 56 ± 4 mg and 67 ± 2 mg of pectins were extracted per g of H4R and H4V-PaL, respectively, compared to 150 ± 10 mg measured in 1 g of non-retted fibres. The amount

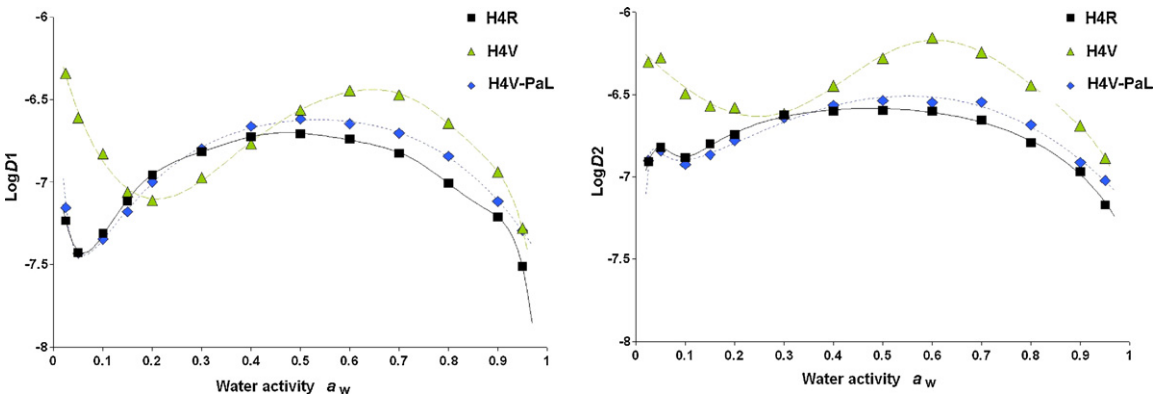

Fig. 2. Effect of retting and enzyme treatments on water diffusion coefficients in technical fibres. D_1 and D_2 were calculated for short or long times (see Section 2). Untreated, H4V, retted H4R, PaL treated H4V-PaL.

of total sugars extracted with H_2O from H4V-PaL was higher (40 ± 5 mg/g) than from H4R (23 ± 4 mg/g), indicating that the few hours of enzyme treatment was not as efficient as the retting fungi to remove the contaminating cortical tissues.

Interestingly, the HGA amount in EDTA was lower in H4V-PaL (7 ± 1 mg/g) than in H4R (13 ± 3 mg/g) for a similar amount of RG-I (20 ± 3 mg/g), indicating that the division process was not totally similar in H4R and in H4V-PaL. In H4R, some HGA that were initially located in the primary wall/S1 layer of fibres were possibly weakened during the retting step.

3.4. Water sorption

3.4.1. Kinetics aspects and diffusion coefficient determination

As the diffusion coefficients were very highly dependent on water activity (a_w), they were semi-logarithm scaled (Fig. 2A and B). Interestingly, the behaviour of H4R and H4V-PaL was quite similar for both D_1 and D_2. For low a_w, the decrease of D (more pronounced for D_1) was attributed to an anti-plasticizing effect of water as previously indicated for green flax samples (Alix, Philippe, et al., 2009), involving intra and inter hydrogen bond formation between free hydroxyl groups of polysaccharides and water molecules, but also the water cluster formation around ionic groups.

As a_w increased up to 0.5–0.6, water was reverted to its role as a typical plasticizer with higher mobility of the dissolved molecules. The D increase was the highest for H4V and both D_1 and D_2 became larger for H4V-PaL than for H4R. Above this a_w critical value, the diffusivity decreasing was due to water molecule clustering.

As seen above, the structural differences between H4V and H4R or H4V-PaL samples originated from (i) the surface with the contaminating cortex tissues and (ii) from the inter-fibre cements. The fact that H4R and H4V-PaL samples exhibited the lowest D_1 and D_2 values within the narrowest range a_w might be due to the lowest diameter of the technical fibre, which made the negative sites, on which water could be transitory immobilized, more accessible. On the other hand, the longer term water anti-plasticizing behaviour of H4V might originate from the presence of the hydrophobic cuticle that reduces the penetration of water into the cortex tissues enriched in pectins.

3.4.2. Isotherms

The water sorption isotherm curves (Fig. 3) were decomposed into three components characterized by the parameters defined in the Park equation (see Section 2 and Table 2).

The former parameter A_L, corresponding to a mono-layer of water at the surface of the fibres (concave part of the curve; $a_w < 0.15$), is dependent on the ratio perimeter/surface of the sample, as well as the textural aspect of the surface. Hence, the A_L value was highest in the cases of H4R and H4V-PaL which consisted of the most divided technical fibres. The slightly higher value observed for H4R in comparison with H4V-PaL was expected due to the heterogeneity of the retting, with the presence of elementary fibres with a higher perimeter/area ratio. In the case of H4V, the Langmuir contribution was insignificant. These fibres were still organized in bundles, so as the ratio perimeter/surface was the highest. Besides, the bundle was surrounded by a pectic gel like layer which masked the surface mesopores of the elementary fibres. According to Morvan et al. (1990), such a layer would contribute to the higher cation exchange capacity of H4V (200–250 mequiv. g^{-1})

Table 2
Parameters of the Park's equation for the non retted green (H4V), retted (H4V) and PaL treated (H4V-PaL) samples. A_L and b_L consisted of Langmuir capacity and affinity parameters; k_H is the Henry's solubility constant; K_a is the equilibrium constant for the clustering reaction and n the mean number of water molecules per cluster. For comparison with vapour sorption data, the % of water absorbed in liquid water is reported as Q_w. H4V, H4R, H4V-PaL corresponded to green, retted and pectate lyase treated technical fibres.

	H4V	H4R	H4V-PaL
A_L	1×10^{-3}	1.3	1.0
b_L	1×10^{-3}	37	65
k_H	13	11	13
K_a	9.5×10^{-13}	2.4×10^{-11}	1.5×10^{-13}
n	11	10	12
Q_w (in liquid water)	$125 \pm 5\%$	$85 \pm 5\%$	$82 \pm 4\%$

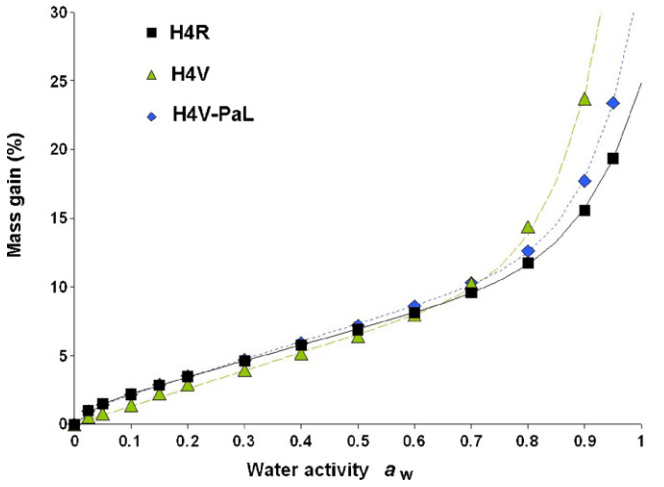

Fig. 3. Water sorption isotherm curves for technical fibres (untreated, H4V, retted H4R, PaL treated H4V-PaL).

Table 3a
Chemical composition of pectins and hemicelluloses released after the chemical extractions of the pre-treated fibres.

mg/g dry fibres	EH extract		EOH extract	
	H4R	H4V-PaL	H4R	H4V-PaL
Ara	2.8 ± 0.4	3.3 ± 0.3	0.8 ± 0.2	0.4 ± 0.1
Gal	29.8 ± 1.8	31.8 ± 2.0	12.6 ± 2.5	12.2 ± 1.2
Rha	5.3 ± 0.5	5.7 ± 0.4	1.7 ± 0.4	1.7 ± 0.1
GalUA	10.8 ± 2.3[2]	7.4 ± 0.6	5.2 ± 1.3[1]	8.5 ± 0.7
Glc	4.1 ± 1.3	5.8 ± 1.6	15.0 ± 1.7[1]	9.7 ± 0.3
Man	2.0 ± 1.3	1.4 ± 0.5	12.2 ± 1.8	9.4 ± 0.4
Xyl	1.2 ± 0.2	2.6 ± 0.4	6.2 ± 1.3	4.7 ± 1.9

NCPs were successively extracted with HCl (EH) and NaOH (EOH). Arabinose (Ara), galactose (Gal), rhamnose (Rha) and galacturonic acid (GalUA) were the main sugars contained in pectins. The sugars specific to hemicellulosic compounds were glucose (Glc), mannose (Man), xylose (Xyl), fucose (Fuc) and glucuronic acid (GlcU). H4R, H4V-PaL corresponded to retted and pectate lyase treated technical fibres.
[1,2]Significantly different from the value of H4V-PaL (Student's test $P < 0.01$ or 0.05, respectively).

Table 3b
Identification of the NCPs in EH and EOH extracts from H4R and H4V-PaL fibres. Comparison of H4R and H4V-PaL with H3R and Oliver (the two later from Alix, Goimard, et al., 2009).

mg/g dry fibres	H3R	H4R	H4V-PaL	Oliver
EH-RG-I	29.0 ± 4.0	43.0 ± 2.6	46.4 ± 3.1	41.5 ± 4.5
EH-HGA	5.0 ± 1.5	5.6 ± 1.8	1.7 ± 0.6	19.5 ± 3.5
Gal/Rha	6.7 ± 0.7	5.6 ± 1.2	5.6 ± 0.8	6.2 ± 1.0
EH-GM	3.3 ± 1.0	4.0 ± 2.5	2.8 ± 0.1	6.7 ± 4.5
OH-RG-I	12.3 ± 2.0	16.7 ± 3.0	16.1 ± 1.3	14.0 ± 2.5
Gal/Rha	12.5 ± 1.0	7.4 ± 2.4	7.2 ± 1.0	13.3 ± 1.5
OH-HGA	5.7 ± 1.0	3.6 ± 1.6	6.8 ± 0.7	6.1 ± 1.0
OH-GM	25.8 ± 3.5	24.4 ± 3.5	18.7 ± 0.7	34.2 ± 4.2

The amount of homogalacturonan (HGA) was calculated as GalUA–Rha contents in Table 4 The amount of rhamnogalacturonan of type I (RG-I) was calculated as (2 × Rha) + Gal + Ara. The amount of glucomannan (GM) was calculated from Van Hazendonk et al. (1996) reporting that in case of flax, Glc = Man/1.5. H4R, H4V-PaL corresponded retted and pectate lyase treated technical fibres. H3R was cultivated in 2003, dew-retted and scutched (Alix, Goimard, et al., 2009).

compared to that of H4R (~100 mequiv. g^{-1}) and might explain the highest value of water adsorption Q_w of H4V measured in liquid water.

On the other hand, b_L made it possible to distinguish H4V-PaL whose value was the highest. More negatively charged pectins exhibiting high affinity for water molecules might remain at their surface.

There was almost no difference for k_H no matter the samples, because the secondary wall (that should not be affected by retting and enzyme treatment) constituted the main part of the total volume in which the water molecules were randomly transported (Henry's type process $0.15 < a_w < 0.60$). Nevertheless, in the H4R sample, the value of k_H was slightly lower. Also, the equilibrium constant K_a, which characterizes the formation of water clusters on polar groups within the cell-wall micro-voids, was the highest in H4R while the number n of aggregates was the least. Such a specific behaviour of H4R might be related to the dew retting process, alternating periods of watering and drying over 7 weeks. Some cell-wall components might have been hydrolyzed/solubilized (see above, the surface content of pectins). On the other hand, drying periods in low humidity environment might impact the polysaccharide-chain mobility and consequently the plastifying effect of water (see also the lower D_1 and D_2 values, Fig. 2, when $a_w > 0.3$, indicating a slower diffusion of water for H4R than for H4V-PAL). This impact would disappear in liquid water (as indicated by Q_w in Table 2).

Figs. 2 and 3 pointed out the highest differences between non retted and treated samples and similarities between H4R and H4V-PaL. Nevertheless, studying in detail kinetics and isotherms makes it possible to approach subtle differences in sorption mechanisms in H4R and H4V-PaL. Stamboulis, Baillie, and Peijs (2001) pointed out the impact of steam or water heating in an autoclave on water vapour sorption which could be attributed to differences in the morphological features of fibres. Further studies have to be run to understand the particular behaviour of H4R when the water vapour activity is increased.

3.5. Characterization of elementary fibres

3.5.1. Chemical composition of H4R and H4V-PaL

After pre-treatments (boiling water and EDTA), the mass of H4R and H4V-PaL mainly originated from the secondary wall (Morvan et al., 2003). The amounts of cellulose (83–85%) and NCPs (10–11%) were similar in H4R and H4V-PaL and not many significant differences appeared in their sugar composition (Table 3a). Interestingly, the percentages of GalUA were opposite in both EH and EOH extracts for H4R and H4V-PaL. If their role in incrusting matrix (EH extract) is recognized, not many papers have reported the presence of galU in structural polymers strongly associated with cellulose (e.g. Girault, His, Andème-Onzighi, Driouich, & Morvan, 2000; Gurjanov et al., 2008; Mooney et al., 2001) and their roles remain to be investigated (Alix, Goimard, et al., 2009). Also the amount of Glc was the highest in the EOH extract of H4R. These data underlined that the two treatments were not totally similar and that subtle structure differences might contribute to the slight differences in the mechanical and sorption behaviour.

From these data, we estimated the amounts of the main NCPs and compared them to those previously reported (Alix, Goimard, et al., 2009) for the target Hermes H3R fibres characterized by high tensile modulus and breaking strength, and for the fibres of Oliver, a winter linseed variety that displayed moderate tensile properties (Table 3b). The aim of the comparison is to facilitate the understanding of what could be the relationship between fibre composition/structure and tensile properties. The amount of pectins in EH (including both RG-I and HGA) of H4R and H4V-PaL (49 and 48 mg/g dry fibres, respectively) was lower than in Oliver (61 mg/g dry fibres) but relatively high compared to H3R (34 mg/g dry fibres). In EOH, the pectin content was about similar in all varieties (18–23 mg/g dry fibres). In both EH and EOH extracts, they mainly consisted of RG-I and their value in H4R and H4V-PaL was closer to that of Oliver than the one of H3R. The ratio of galactose to rhamnose, representing the mean side-chain length of β-1,4 galactan, increased only slightly from EH to EOH in H4R and H4V-PaL. As this ratio in EOH was lower than 10, it is unlikely that such short side-chains interact with cellulose by hydrogen bonding (Zykwinska et al., 2007). The lack of such bonding would reduce the strength of the interface between the cellulose and pectic matrix (Alix, Goimard, et al., 2009), which might represent one factor of the low tensile properties of the 2004 samples (see below).

The ratio between the amount of coating GM in EOH and the level of matrix pectins in EH was rather low (<0.6) in H4R and H4V-PaL samples. This ratio was close to that of Oliver, but significantly less than H3R (0.75). These results suggest that the content of the pectic matrix was too large for the glucomannans to ensure all the cross-linkings between cellulose microfibrils. This might be a second factor explaining the sample's moderate tensile properties. The chemical differences between H3R, on the one hand, and H4R or H4V-PaL, on the other hand, would originate from the growth conditions that influenced the filling (e.g. Chemikosova, Pavlencheva, Gur'yanov, & Gorshkova, 2006; Milthorpe, 1945; Ripoll et al., 1993), and the NCP remodelling of the deposited layers (Gorshkova & Morvan, 2006).

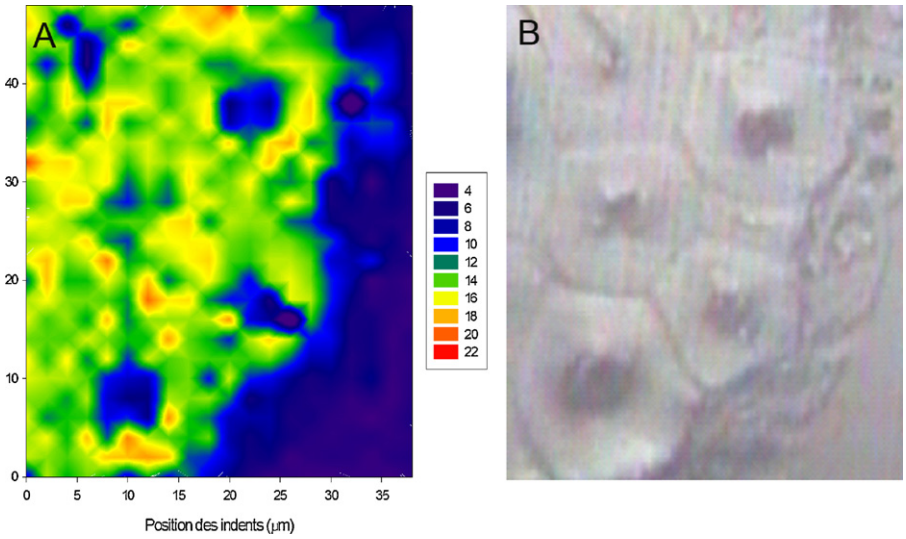

Fig. 4. Indentation on a section of H4V flax fibres; 20 × 20 matrix with 1 μm between each indent. (A) False colour cartography of the Young's modulus ($E-n$). (B) Micrograph of fibre section after nanoindentation experiments.

3.5.2. Tensile properties of the elementary fibres

As for the mechanical tests performed on technical fibres, a large scattering of tensile values were observed when the experiment was carried out on elementary fibres (Table 4). Interestingly, H4V-PaL fibres displayed the lowest variability for both parameters E and σf. This consisted of the only mechanical difference that made it possible to distinguish between the three samples. Whatever the sample, H4V, H4R or H4V-PaL, there were no significant differences between the strength (σf) and stiffness (E) parameters (conversely to the tendency observed for the technical fibres). Thus, PaL-treatment which preserved the mechanical behaviour of the elementary fibres again proved to be as a good candidate to substitute dew-retting.

It has been previously reported (Charlet et al., 2007) that elementary fibres isolated from the same Hermes variety (named H3R) exhibited high tensile properties (i.e. $E = 68 \pm 36$ GPa and $\sigma f = 1454 \pm 835$ MPa) and low failure strain (2%). The values of the failure strain of the three samples, H4V, H4R or H4V-PaL, were larger than that of H3R, suggesting that the higher level of matrix pectins might favour the sliding of cellulose microfibrils. On the other hand, the two other parameters E and σf of all three samples were significantly lower indicating that their elementary fibres were less stiff and weaker than H3R. The tensile data of H4 elementary fibres were situated in the lowest range of values which had been reported for similar average diameters and clamping distance (e.g. Bos et al., 2002). Because the PAL-enzymatic treatment of H4-V that was run in controlled conditions did not lead to better values than H4R and H4V, over-retting would not be the main explanation. Alternatively, the moderate tensile values might be attributed to the impact of growth conditions (soil, agricultural practice or weather) on structure of the fibres as pointed out by Sharma and Faughey (1999) or Norton et al. (2006). Using a multicomponent analysis, including 26 varieties, 2 year cultivation and 2 nitrogen treatments, the latter authors concluded the importance of the season for both the fineness and strength of all varieties. Hermes was recognized as a variety with a high fibre percentage but having coarse fibres, displaying moderate mechanical properties. From a chemical and structural point of view, Alix, Goimard, et al. (2009) suggested that not only the percentage, crystallinity and orientation of cellulose were important for high tensile performance, but also the diversity of interface/interphase that were established between the pectic matrix and the cellulose microfibrils. As noted above, the tensile properties, the chemical composition and composite structure were very different between the three H4 samples and H3R. Studies with more varieties and growth conditions have to be performed to test the Alix hypothesis.

Table 4
Mean tensile properties of elementary fibres. The gauge length was 10 mm and the cross-head displacement rate 1 mm min^{-1}. σf, εf, E, corresponded to the stress, strain at the sample failure (f) and Young's modulus respectively. The diameter values corresponding to the mean of those of the tested samples (number tested = n) were about similar as the one estimated by microscopy for several hundred fibres. H4V, H4R, H4V-PaL corresponded to green, retted and pectate lyase treated technical fibres. According to Student's test ($P < 0.05$–0.1) no significant difference was observed between the samples.

	H4V	H4R	H4V-PaL
σf (MPa)	670 ± 315	670 ± 320	635 ± 245
εf (%)	3.5 ± 1.1	3.1 ± 1.1	3.4 ± 1.1
E (GPa)	36 ± 15	37 ± 14	36 ± 10
⌀ (μm)	17 ± 5	16 ± 4	17 ± 4
N	58	38	38

3.5.3. Investigation of mechanical properties of flax fibres by using nanoindentation

Fig. 4 shows the colourized cartography of the H4V modulus ($E-n$) together with a fibre picture after the nanoindentation test. A good correlation could be noticed between the localization of the fibres and high $E-n$ values, the lowest values corresponding to the resin or lumen areas. Due to the important part of the secondary wall within the fibre section, the nanoindentation results were likely to be associated with the mechanical properties of the secondary wall and especially with the S2 layer. Our cartography also highlighted high $E-n$ in the junctions of fibres.

The $E-n$ values were calculated using a Poisson's ratio of 0.35 (Baiardo, Zini, & Scandola, 2004) and averaged over an indentation depth of 100–110 nm from a minimum of 272 indents. They were found within the range 14 and 22 GPa, being significantly larger for H4V-PaL (20.5 ± 1.8 GPa) than for H4V (15.2 ± 1.4 GPa) and H4R (17.7 ± 1.5 GPa).

Compared to H4V, $E-n$ significantly increased after treatments, especially in the case of H4V-PaL. The difference might result from some physico-chemical processes that happened during the enzymatic treatment and during dew-retting. As underlined above during dew-retting, water could solubilize small cell-wall components (called extractibles) while drying might induce inter-

molecular cross-linking. Although, PaL treatment time was short, it was performed in a liquid buffer; consequently, extractibles might also be eluted, which were not necessarily the same as the dew-retting ones. Meszaros, Jakab, and Varhegyi (2007) have shown, in wood, the impact of extractibles in the mobility of lignin and hemicellulose and consequently on the cellulose rigidity and fibre $E-n$. Similar dynamical mechanical measurements could be interesting to take to test whether or not such a phenomenon also occurred in fibres when they had stayed in water enough time.

Alternatively, part of the $E-n$ differences might originate from the samplings, due to the natural variability of the structural properties of fibres among stems, parts of stem or even within one bundle. Although the nanoindentation experiments were performed on several fibres, the number of indented sections was smaller than the number of fibres tested by tensile tests. This might partly explain why such a difference between module values of H4R and H4PaL was not found after two tensile tests on elementary fibres where E values were almost similar for both samples.

However, it is difficult to compare the nanoindentation and tensile results due to the measurement scale differences and to the sollicitation modes. In a previous work, Bourmaud and Pimbert (2008) have pointed out differences between nanoindentation and tensile tests using hemp and sisal fibres; nanoindentation $E-n$ was estimated at 12.1 ± 2.3 GPa and 8.5 ± 1.7 GPa for hemp and sisal fibres, respectively. In the same study, the tensile elastic modulus was calculated at 44.5 ± 19.1 GPa for hemp fibres and 25.0 ± 12.9 GPa for sisal. On the one hand, we might expect larger values for the modulus within the restricted region of the secondary wall submitted to nanoindentation than when submitting the total fibre, including its primary wall and lumen, to tensile test. On the other hand, these differences could be due to the fact that a nanoindentation test is closer to a compressive test than a tensile experiment. Moreover, due to the inclination on faces of the Berkovich-type indenter (Gindl & Schoberl, 2004); with this particular geometry, the wall was loaded at an angle of approximately 25°. Consequently, the resulting three-dimensional stress was not only governed by the longitudinal modulus, but also affected by the transverse modulus, inducing an underestimation of the longitudinal modulus. More studies would have to include compression and flexion experiments in addition to tensile and nanoindentation tests.

4. Conclusions

The main conclusion, deriving from all the data collected to compare the impacts of dew retting and enzymatic treatments points out the efficiency of PaL treatment to mime dew-retting. As seen by microscopy, similar bundle division was reach and importantly the mechanical properties of technical fibres were preserved or possibly improved. On the other hand, PaL-treatment preserved the structure of the elementary fibres since (i) the tensile values, (ii) the chemical composition and the sorption behaviour of H4R and H4V-Pal were relatively similar. This was expected since PaL treatment had been chosen to specifically eliminate the pectins of surrounding tissues (enriched in HGA), but only partly those of the fibre junctions (enriched in RG-I) (Jauneau et al., 1992). Interestingly, the scattering range of the tensile values of elementary fibres were the lowest for H4-PaL. This might be related either to a facilitated isolation of fibres or to a better structuration of the secondary-wall, which might explain the higher value of $E-n$. Nanoindentation data have to be confirmed. Another approach, such as DMA, might be worthwhile to check the impact of watering and drying, i.e. removing or cross linking extractibles, and possibly to specify the subtle differences detected between H4V-PaL and H4R during sorption experiments.

A secondary conclusion originates from the comparison of the biochemical data obtained from Hermes cultivated either in 2003 or in 2004. It deals with a global impact of the environment (soil, agricultural practice and/or weather).

The H4 fibres might be not fully maturated with higher porosity than usual (Charlet, 2008), suggesting that the last step of cell differentiation did not occur, i.e. the fibre structuration/dehydratation (as defined in Morvan et al., 2003) and the reduction of fibre diameter (in the middle part of the stem) did not happen. The sugar analyses pointed out an excess of matrix pectin compared to the amount of coating glucomannan polymers, as well as a lack of interactions between galactans and cellulose. This would lead to a weak interface between the cellulose microfibrils and the pectin matrix and consequently reduce tensile properties of the fibres (Alix, Goimard, et al., 2009). Sharma and Faughey (1999) found that variation between varieties could be related to non cellulosic components, the lower the levels of NCPs, the finer the fibres. Variation in fibre structure may occur as a result of variation in weather during certain key physiological periods such as when the stems are laid down and during retting including stand retting (Norton et al., 2006; Sampaio, Bishop, & Shen, 2005). Such effects of the dessication time require more in-depth study as it is not yet clear how they might be beneficial, if at all, for the commercial value of technical fibres.

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